

AMENDMENTS TO THE SPECIFICATION

Please replace paragraph [0008] with the following amended paragraph:

[0008] Proinflammatory cytokines such as interleukin-1 (IL-1), and tumor necrosis factor- α (TNF- α) produced by cells at the injured or infected site stimulate the endothelium to produce chemokines such as interleukin-8 (IL-8) and integrin binding ligands such as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs) on the surface of the endothelial cells opposite the basal lamina. The chemokines are held on the surface of the endothelial cells opposite the basal lamina where the chemokines interact with chemokine receptors on the surface of the rolling leukocytes. This interaction, in turn, triggers the activation of molecules called integrins on the surface of the leukocytes. Integrins are a family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix proteins of the basement membrane or to ligands on other cells. Integrins are composed of large α and small β subunits. Mammalian integrins form several subfamilies sharing common β subunits that associate with ~~difference~~ different α subunits. \exists_2 integrins (the “CD-18 family”) include four different heterodimers: CD11a/CD18 (Lymphocyte Function-Associated Antigen-1 (LFA-1)); CD11b/CD18 (Mac-1); CD11c/CD18 (p150,95), and CD11d/CD18. The most important member of the \exists_1 integrin subfamily on leukocytes is Very Late Antigen 4 (VLA-4, CD49d/CD29, $\forall_4\exists_1$). Activation of these integrins by chemokines enables the slowly rolling leukocytes to “arrest” and strongly bind to the endothelium’s ICAMs, VCAMs, and other integrin binding ligands of the endothelial cells, such as collagen, fibronectin, and fibrinogen. Once bound to the endothelial cells, the leukocytes then flatten and squeeze between the endothelial cells to leave the blood vessels and enter the damaged tissue through a process termed “transmigration.” Transmigration is thought to be mediated by platelets, endothelial cell adhesion molecule-1 (PECAM-1), junctional adhesion molecule (JAM), and possibly CD99, a transmembrane protein.

Please replace paragraph [0060] with the following amended paragraph:

[0060] In another embodiment of the present invention, channel 26 includes a plurality of leukocyte migration mediators disposed therein. Preferably, the plurality of leukocyte migration mediators comprises at least one first leukocyte migration mediator and at least one second

leukocyte migration mediator, wherein the at least one first and the at least one second leukocyte migration mediators are different from one another. The leukocyte migration mediators are disposed in channel 26 so as to form a surface concentration gradient along a ~~longitudinal~~
longitudinal axis of chamber 14 in increasing concentration from first well 20 18 to second well 22.

Please replace paragraph [0064] with the following amended paragraph:

[0064] Because device 10, or elements of device 10, may match the footprint of an industry standard microtiter plate, an advantage of device 10 is that device 10 may be used to conduct multiple assays simultaneously in the same device, and to high throughput screen various test agents. In one preferred embodiment, as illustrated in Fig. 5, the first well regions 16 and the second well regions 20 of the respective ones of the plurality of chambers 14 are disposed relative to one another to match a pitch of a standard microtiter plate. Taking P to designate a pitch between respective wells 18/22, the wells may be disposed relative to one another to match a pitch of one of a 24-well microtiter plate, a 96-well microtiter plate, a 384-well microtiter plate, 768-well microtiter plate and a 1536-well microtiter plate. By way of example, in the configuration of chambers 14 as shown in Fig. 5, pitch P will be set to about 9 mm. Preferably, device 10 itself fits in the footprint of an industry standard microtiter plate. As such, device 10 preferably has the same outer dimensions and overall size of an industry standard microtiter plate. By way of example, in the configuration of device 10 as shown in Fig. 6, device 10 comprises 48 chambers designed in the format of a standard 96-well plate, such that the respective wells 18/22 are disposed relative to one another to match a pitch of a standard 96-well microtiter plate with each well fitting in the space of each well of the plate. In this embodiment, 48 experiments can be conducted. Alternatively, as seen in Figure 6A, chambers 14 may be disposed relative to one another to match a pitch of a standard microtiter plate. In this alternative embodiment, chambers 14 are sized so that a chamber 14 fits in the area normally required for a single well of a standard microtiter plate. For example, in this embodiment, device 10, designed in the footprint of a 96-well microtiter plate configuration, has 96 chambers and therefore allows 96 experiments to be performed. By conforming to the exact dimensions and specification of standard microtiter plates, embodiments of device 10 would advantageously fit into existing infrastructures of fluid handling, storage, registration and detection. Device 10 is also conducive

to high throughput screening as it allows robotic fluid handling and automated detection and data analysis. The use of robotic and automated systems also decreases the amount of time to prepare and perform the assays and analyze the results of the assays. In addition, by using automated systems, the use of device 10 decreases the occurrence of human error in preparing and performing assays and analyzing data. Moreover, because the size of the wells 18/22, or the size of an entire chamber 12, of device 10 matches the size of a well of a microtiter plate, the number of leukocytes needed to perform an individual assay range from only about 103 to about 106. This allows for the study of rare leukocyte populations, such as basophils or certain lymphocyte subsets. In addition, large amounts of test agents, such as inhibitors and promoters of leukocyte migration, need not be used in order to conduct assays monitoring the effect of these agents on leukocyte migration.

Please replace paragraph [0099] with the following amended paragraph:

[0099] With respect to portions of chamber 14, in one embodiment, well regions 16 and 20 are vertically offset with respect to one another in a test orientation of device 10. In a preferred embodiment, well regions 16 and 20 are horizontally offset with respect to one another in a test orientation of device 10. Wells 18 and 22 of respective well regions 16 and 20 of each chamber 14 are not limited in their configuration to any particular three dimensional contour, it being only required that they be adapted to receive a fluid therein, preferably a sample comprising leukocytes. Preferably, wells 18 and 22 are configured such that they substantially define circles in top plan views thereof, as shown by way of example in Figs. 1-6. However, other contours in the top plan view of a given well is within the scope of the present invention, as readily recognized by one skilled in the art. Where the wells define circles in top plan views thereof, and where, the well regions are disposed relative to one another to match a pitch of a standard 96-well microtiter plate, the pitch P is set to be equal to about 9 mm, and the diameter D_w of a top plan contour of the wells is set to be equal to about 6 mm. In such a case, length L of each channel 26 is equal to about 3 mm. As shown in particular in Fig. 2, wells 18 and 22 are defined in part by respective through-holes 18 and 22 18¹ and 22¹ in top member 30, and in part by an upper surface U of support member 28. In particular, the sides of each well 18 and 22 are defined by respective walls of the through holes ~~18 and 22~~ 18¹ and 22¹ in the top member 30, and

the bottoms of wells 18 and 22 are defined by a corresponding portion of the upper surface U of support member 28.

Please replace paragraphs [0112]-[0013] with the following amended paragraphs:

[0112] In addition to the various immobilization schemes, further assay design flexibility centers around the leukocyte migration mediators or other biomolecules present in channels 24 26. For example, in one embodiment, one type of leukocyte migration mediator is present in each channel 24 26 at the same concentration. In another embodiment, one type of leukocyte migration mediator is present in each channel 24 26 at differing concentrations. In another embodiment, different leukocyte migration mediators are present in each channel 24 26. In another embodiment, there is a mixture of leukocyte migration mediators in each channel 24 26. Each channel 24 26 may have the same mixture or a different mixture. When the mixture is the same, the ratios or concentrations of the different leukocyte migration mediators may be different in each channel 24 26.

[0113] Likewise with respect to compounds, such as drugs or test substances, the present invention provides flexibility in assay design. For example, in one embodiment a single compound is present in all channels 24 26 at the same concentration throughout. In another embodiment, the same compound is present in all the channels 24 26 but each channel 24 26 has a different concentration of that compound. In another embodiment, each channel 24 26 has a different compound. In another embodiment, there is more than one compound. When there is more than one compound, each channel 24 26 may have the same mixture of compounds or may have a different mixture of compounds. Further, when the mixtures of the compounds are the same, each channel 24 26 may receive a different concentration of that mixture. Yet, even further, each channel 24 26 may receive the mixture of the compounds, with each channel 24 26 having a different ratio of compounds to each other.

Please replace paragraph [0116] with the following amended paragraph:

[0116] A master of the device according to the present invention is made using photolithography. A silicon substrate is patterned based on a negative pattern of the top member using a suitable photoresist. Thereafter, polydimethyl siloxane (PDMS) is poured on top of the

master and placed under vacuum in order to extract air bubbles therefrom. The thus poured PDMS layer is allowed to cure in an oven at about 30°C for about 17 hours. Thereafter, the device is washed thoroughly with 2% Micro-90 (a product of International Products Corp.), rinsed for 10 minutes at 70° C in "Sonic Bath," and rinsed with de-ionized water, followed by a rinsing with 100% ethanol. The PDMS layer is then dried under nitrogen. At the same time, a pre-cleaned glass slide, such as a rectangular one having dimensions of about 4.913 +/- 0.004 inches (in.) by about 3.247 +/- 0.004 in. and a thickness of about 1.75 millimeters (mm), mm, is washed three times with ethanol and twice with methanol. Preferably, the surfaces of the PDMS layer and the glass slide to be bound together are both plasma oxidized for about 84 seconds. The PDMS layer and the glass slide are then pressed together using forceps to squeeze out air pockets therebetween. In this manner, a fluid-tight, conformal contact is established between the PDMS layer as top member and the glass slide and as support member. In addition, by virtue of PDMS having been used as the top member material, the conformal contact between the PDMS layer and the glass slide is reversible.

Please replace paragraph [0121] with the following amended paragraph:

[0121] In order to characterize the rolling velocity of the leukocytes at a particular time, an image obtained using the method described in part C is used measure the distance the leukocytes traveled during the exposure time of the image. To determine rolling velocity (V), the following formula is used:

$v = c(l_{time} / t_{exposure})$ where
c: conversion conversion factor for determining the actual distance the cells traveled.

This factor may vary from image to image.

l_{time} : the length of the leukocytes migration in the captured image.

$t_{exposure}$: the exposure time of the image.

Preferably $t_{exposure}$ is 100 milliseconds (ms) when the flow rate is about 0.1 dynes/cm² to about 20 dynes/cm².

Please replace paragraph [0123] with the following amended paragraph:

[0123] 20 μ l of water are pipetted in the first well of the chamber of the device fabricated according to method disclosed in section I. Microcapillary action draws the water into the channel. After ensuring no air bubbles are inside the channel, an additional 10 μ l of water

are pipetted out in the second well of the chamber. After 15 minutes pass and the hydrostatic pressure equalizes, 10 μl of P-Selectin with a concentration of 50 $\mu\text{g}/\text{mL}$ (obtained from R&D Systems, catalog #ADP3) is pipetted in the first well and 10 μl of ICAM-1 with a concentration of 50 $\mu\text{g}/\text{mL}$ (obtained from R&D Systems) is simultaneously pipetted in the second well. The device is incubated for two hours at room temperature in a dish with a cover in order to keep the wells from drying out. After the incubation, the channel is washed four times using 0.1% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS). After this last wash, all the liquid inside the wells is pipetted out leaving only liquid in the channel. 20 μl of 0.1% BSA in PBS is added to the first well and 10 μl of BSA in PBS is added to the second well. After 15 minutes pass and the hydrostatic pressure equalizes, neutrophils isolated from part A in 60 μl of media are added to the first well of each chamber (about 10^3 to about 10^6 cells per well of a 24 well plate, in volume of 60 μl of media per well) (non-labeled and fluorescently labeled monocytic cell lines-U937 and THP-1 as well as primary leukocytes may also be used). As seen in Figure 7, it is preferred that 40 μl - 60 μl of media be used to generate the range of flow velocity under normal physiological conditions (about 0.1 dynes/cm² to about 20 dynes/cm²).

Please replace paragraph [0129] with the following amended paragraph:

[0129] In order to characterize the rolling velocity of the cells at a particular time, an image obtained from the method described in part C is used to measure the distance the leukocytes traveled during the exposure time of the image. To determine rolling velocity (V), the following formula is used:

$$v = c(l_{\text{time}} / t_{\text{exposure}}) \text{ where}$$

c: conversion conversion factor for determining the actual distance the cells traveled.

This factor may vary from image to image.

l_{time} : the length of the leukocytes migration in the captured image.

t_{exposure} : the exposure time of the image.

Preferably t_{exposure} is 100 ms when the flow rate is about 0.1 dynes/cm² to about 20 dynes/cm².

Please replace paragraph [0133] with the following amended paragraph:

[0133] Neutrophils are isolated according to the method disclosed in section II IV, part A.

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